Effect of Vitamin B₂ Deficiency on Rat Liver Dihydropyrimidine Dehydrogenase Activity

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Summary Effect of vitamin B₂ deficiency on rat liver dihydropyrimidine dehydrogenase was investigated. It was found that the purified enzyme contains 2 mol flavin per molecule, which consists of equal proportions of flavin-adenine dinucleotide (FAD) and riboflavin 5'-phosphate (FMN). When rats were fed on a vitamin B₂-deficient diet for 5 weeks, dihydropyrimidine dehydrogenase activity in the liver was diminished, followed by a decrease in enzyme concentration. Moreover, the addition of exogenous FAD or FMN did not restore the activity. Thus endogenous flavin may regulate the enzyme half-life or synthesis. Lowering of dihydropyrimidine dehydrogenase activity in the livers of rats fed on a vitamin B₂-deficient diet did not affect the uridine, uracil and Σ UMP (the sum of acid soluble uracil 5'-nucleotides) pool in liver.

Key Words pyrimidine metabolism, dihydropyrimidine dehydrogenase, flavin, vitamin B₂ deficiency

Pyrimidine degrading enzymes, dihydropyrimidine dehydrogenase [EC 1.3.1.2], dihydropyrimidinase [EC 3.5.2.2], β -ureidopropionase [EC 3.5.1.6], aminobutyrate aminotransferase [EC 2.6.1.19], and D- β -aminoisobutyrate-pyruvate aminotransferase [EC 2.6.1.40] are mainly present in liver (1–3), and pyrimidines are mainly degraded in liver. Dihydropyrimidine dehydrogenase is the rate-limiting enzyme in the uracil catabolic pathway (1). The enzyme activity increases during differentiation after birth (1). It seems that dihydropyrimidine dehydrogenase activity decreases when cells require many pyrimidine bases during growth. Moreover, the enzyme degrades 5-fluorouracil, which is an antitumor drug, and the level of the enzyme affects the antitumor efficacy of 5-fluorouracil (4). 5-Fluorouracil has strong side effects in patients with no dihydropyrimidine dehydrogenase activity (5). Dihydropyrimidine dehydrogenase purified from rat liver to homogeneity by Shiotani and Weber (6) was reported to contain flavin. How-

ever, the role of flavin is still unknown. In this study, we found flavin may regulate the half-life or the synthesis of the enzyme, since the administration of a vitamin B₂-deficient diet decreased the concentration of dihydropyrimidine dehydrogenase in rats.

MATERIALS AND METHOD

Chemicals and animals. [6-³H]Uracil was obtained from New England Nuclear, U.S.A. Vitamin B₂-deficient diet was purchased from Oriental Yeast, Tokyo. FAD, FMN, and NAD⁺ were the products of Sigma Chemicals. Immune Blot Assay Kit was from Bio-Rad Laboratories. All other chemicals were purchased from Nakalai Tesque, Kyoto; rats (Wistar) and rabbits were obtained from Japan SLC.

Enzyme assays. Dihydropyrimidine dehydrogenase activity was determined by measuring uracil reductase activity at 37°C for 20 min according to the method of Naguib *et al.* (7). The standard assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 50 mM 2-mercaptoethanol, 1 mM NADPH, 20 μ M [6-³H] uracil (1.48 MBq/ μ mol), and 50 μ l enzyme in a final volume of 0.5 ml. The method of measuring NADPH consumption at 340 nm was not used, since the added samples interfered with the measuring the enzyme activity because of their absorbance. Tyrosine aminotransferase activity was determined by the method of Granner and Tomkins (8). Glutathione reductase activity was determined by the method of Racher (9).

Tissue and subcellular distribution of dihydropyrimidine dehydrogenase. Tissues were homogenized in buffer A (10 mM potassium phosphate buffer, pH 7.4, 5 mM 2-mercaptoethanol and 2.5 mM MgCl₂). Dihydropyrimidine dehydrogenase activity in crude homogenate was estimated. For the estimation of subcellular distribution, rat liver was cut into small pieces, and homogenized in 5 volumes (v/w) ice-cold medium (5 mM Tris-HCl buffer, pH 7.3, and 0.25 M sucrose) in a Potter-Elvehjem homogenizer. The homogenate was fractionated by the method mentioned previously (10). The following enzyme markers were assayed to assess the purity of the various subcellular fractions: lactate dehydrogenase for cytosol (11); glutamate dehydrogenase for mitochondria (12); catalase for peroxysomes (13); glucose-6-phosphatase for microsomes (14).

Molecular weight determination. Gel filtration was performed according to the method of Andrews (15) with a column of Sephacryl S-300 equilibrated with buffer A. The column was calibrated with the following markers: pyruvate kinase (230,000), catalase (240,000), aldolase (158,000), alcohol dehydrogenase (141,000), and bovine serum albumin (68,000). Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis (16).

Flavin and iron determination. Flavin was released from the purified enzyme in buffer A by heating at 80°C for 15 min in the dark. Total flavin content was determined by the lumiflavin fluorescence method (17). FAD and FMN were

determined by HPLC (Waters M-600 model) with a reverse-phase C18 analytical column (Waters, 5 μ , 0.39 $\times$ 15 cm). FAD and FMN were separated by a linear methanol gradient from 50 mM potassium phosphate buffer, pH 5.6, to 60% methanol for 20 min, and 60% methanol for 10 min (flow rate 0.7 ml/min). Iron content in the enzyme was determined using a Hitachi 180-70 atomic absorption spectrometer, after dialyzing to 10 mM Tris-HCl buffer, pH 7.5.

Uridine, uracil, and Σ UMP pool determination. Freeze-clamped liver tissue was homogenized in 5 volumes of 0.6 M perchloric acid. After centrifugation, the supernatant was neutralized and added to an assay mixture containing 0.2 M Tris-HCl, pH 8.9, 0.5 mM MgCl_2 and 0.015 unit of snake venom phosphodiesterase. After standing at 37°C for 60 min, the reaction was stopped by boiling for 5 min. After treatment with Sep Pak C18 (Waters) and concentration, uridine, uracil and Σ UMP were separated by HPLC using a linear methanol gradient from 50 mM potassium phosphate buffer, pH 5.6, to 20% methanol for 40 min, on the same column as mentioned above, and were detected at 254 nm.

Immunological procedures:

Preparation of antiserum. Purified dihydropyrimidine dehydrogenase (0.2 mg) was emulsified with an equal volume of Freund's complete adjuvant and injected twice into the foot pad of rabbits at 2-week intervals. Two weeks after the second injection, 0.1 mg of the enzyme was injected intravenously. Rabbits were bled 1 week after the final injection. Control serum was prepared from rabbits prior to the first injection of purified enzyme. Immunoprecipitation and immunodiffusion procedures have been described in a previous report (18).

Western blotting. Rat liver was homogenized with buffer A, mixed with an equal volume of sample buffer (20 mM Tris-HCl buffer, pH 6.8, 2% SDS, 40% glycerol and 2% 2-mercaptoethanol) and heated at 50°C for 60 min. Dihydropyrimidine dehydrogenase separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (19) was transferred to a membrane and color-developed with an Immune Blot Assay Kit (Bio-Rad peroxidase development kit).

Administration of vitamin B₂-deficient diet. The composition of vitamin B₂-deficient and control diet is shown in Table 1. Four-week-old rats were given a vitamin B₂-deficient or a control diet for 5 weeks and were examined for vitamin B₂ content, dihydropyrimidine dehydrogenase, glutathione reductase and tyrosine aminotransferase activity in the liver.

Protein measurements. Protein concentration in partially purified enzyme was measured by the Folin-Lowry method (20) and by dye-binding assay using Bio-Rad protein assay in the purified enzyme.

RESULTS

Distribution of dihydropyrimidine dehydrogenase in rat liver

Dihydropyrimidine dehydrogenase, which catalyzes NADPH-dependent

Table 1. Composition of diet.

Ingredients	Control (%)	Vitamin B ₂ -deficient (%)
Casein	25	25
Sucrose + starch ^a	53	53
Soybean oil	6	6
Salt mixture ^b	6	6
Vitamin mixture ^b	2	
Vitamin mixture ^c (riboflavin-free)		2
Cellulose powder	8	8

^aSucrose:corn starch=2:1. ^bSalt mixture and vitamin mixture were purchased from Oriental Yeast. ^cRiboflavin-free vitamin mixture was also obtained from Oriental Yeast.

uracil reductase activity, was mainly present in liver; the specific activity was $0.032 \pm 0.007 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ($n=3$). The enzyme levels in other rat organs in order of decreasing activity in $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ were 0.032 ± 0.007 adrenal, 0.016 ± 0.006 small intestine, 0.010 ± 0.007 brain and 0.004 ± 0.001 kidney ($n=3$). Most rat liver dihydropyrimidine dehydrogenase activity was located in the cytosolic fraction, of which the purity was assessed by the distribution of marker enzymes. NADH-dependent uracil reductase activity was not present in rat liver under our experimental conditions.

Purification and properties

Dihydropyrimidine dehydrogenase was purified from 50 g of rat liver by the following procedure: heat treatment, pH 4.85 precipitation, $(\text{NH}_4)_2\text{SO}_4$ precipitation, DEAE-Sepharose, hydroxyapatite, Sephacryl S-200, ADP-Sepharose, and CM-Sepharose, a modification of the Shiotani and Weber method (6). The active fraction from DEAE-Sepharose column was applied to a hydroxyapatite column ($2.0 \times 15 \text{ cm}$) equilibrated with buffer A and eluted by a linear potassium phosphate gradient from buffer A to 0.5 M potassium phosphate buffer, pH 7.4, 5 mM 2-mercaptoethanol and 2.5 mM MgCl_2 . The active fraction from the ADP-Sepharose 4B column was applied to a CM-Sepharose CL-6B column ($1.0 \times 1.0 \text{ cm}$) equilibrated with 10 mM potassium phosphate buffer, pH 6.0, 5 mM 2-mercaptoethanol and 2.5 mM MgCl_2 (buffer B). The enzyme was eluted from the column with buffer B. The purified enzyme showed homogeneity by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. A typical purification step is shown in Table 2. The yield of purified enzyme was about 2.9% with a 1,317-fold increase in the specific activity. The purified enzyme in buffer A was very unstable and lost 50% of its activity after 24 h. Addition of 25% glycerol, 1% bovine serum albumin or 0.3 M 2-mercaptoethanol stabilized the enzyme for 3 weeks. Molecular weight of the purified enzyme estimated by gel filtration was 207,000 and subunit molecular weight estimated by gel electrophoresis with SDS was 98,000. Dihy-

Table 2. Purification of dihydropyrimidine dehydrogenase from rat liver.

Steps	Total activity (nmol·min ⁻¹)	Specific activity (nmol·min ⁻¹ ·mg protein ⁻¹)	Total protein (mg)	Purification (fold)
Homogenate	712	0.083	8,560	1.0
Heat treatment	656	0.147	4,462	1.8
pH 4.85 supernatant	791	0.328	2,408	4.0
30–50% (NH ₄) ₂ SO ₄ fractionation	784	1.06	737	12.8
DEAE-Sephacryl CL-6B	454	1.79	253	21.6
Hydroxyapatite	454	24.7	18.4	298
Sephacryl S-200	147	41.4	3.55	498
ADP-Sephacryl 4B	34.6	93.5	0.37	1,126
CM-Sephacryl CL-6B	20.8	109.3	0.19	1,317

Dihydropyrimidine dehydrogenase was purified from 50 g of rat liver.

dihydropyrimidine dehydrogenase contained flavin in the ratio 1.8 mol/mol of enzyme. FAD and FMN ratios were 0.76 and 0.70 mol/mol of enzyme, respectively. The enzyme contained 14 mol iron/mol of enzyme. Flavin bound non-covalently to the enzyme, since it was released from the enzyme by heating at 80°C for 15 min. Iron was not removed from the enzyme by dialysis against 4 mM EDTA in 50 mM Tris-HCl buffer, pH 7.4, and 2 mM 2-mercaptoethanol or against lithium-hydroxyquinoline-5-sulfonic acid in 1 M imidazole-HCl buffer, pH 7.0, and 30 mM NH₂OH·HCl.

Inhibition of dihydropyrimidine dehydrogenase activity

Pyrimidine metabolites (β -alanine, β -ureidopropionate, UMP, UDP, UTP, and uridine, 2 mM) other than substrates did not inhibit the enzyme. Uridine did not inhibit purified enzyme activity, in spite of Tuchman's report (21), which claimed non-competitive inhibition of partially purified dihydropyrimidine dehydrogenase by uridine. Inhibitory effects of metal ions in the absence of 2-mercaptoethanol were studied on enzyme partially purified to the Sephadex S-200 step. CuCl₂ (1 mM) and PCMB (5 mM) inhibited enzyme activity. The relative activities to control were 0.08 and 0.42, respectively. These results and the stabilization by 2-mercaptoethanol shows dihydropyrimidine dehydrogenase is an SH enzyme. Other metal ions (ZnCl₂, MnCl₂, FeCl₂, FeCl₃, and CaCl₂, 1 mM) had no effect on dihydropyrimidine dehydrogenase activity.

Effect of vitamin B₂-deficient diet on dihydropyrimidine dehydrogenase activity and uridine, uracil, and Σ UMP pool in rat liver

Increase of body weight in the rats fed on vitamin B₂-deficient diet was slightly inhibited comparing with the rats fed on control diet. As shown in Fig. 1,

dihydropyrimidine dehydrogenase activity in vitamin B₂-deficient rat livers significantly decreased to about 25% of control. Vitamin B₂ content and glutathione reductase activity, which is a flavin enzyme and a marker of vitamin B₂ deficiency, also decreased to about 25%. Tyrosine aminotransferase was not affected by vitamin B₂ deficiency. The effects of supplementary addition of FAD or FMN to the assay mixture upon dihydropyrimidine dehydrogenase activity of vitamin B₂-deficient rats are shown in Table 3. There was no recovery of activity by flavin addition to the liver homogenate of rats fed a vitamin B₂-deficient diet and it was thought that dihydropyrimidine dehydrogenase levels decreased with vitamin B₂ deficiency. Thus the enzyme protein content in liver was analyzed by Western blotting. Antiserum prepared against purified rat liver dihydropyrimidine dehydrogenase was absorbed by dog liver homogenate, since the antiserum showed 2 precipitin lines against rat liver homogenate on double immunodiffusion. Antiserum to rat liver dihydropyrimidine dehydrogenase did not precipitate dog or guinea pig liver enzyme activity. Absorbed antiserum showed a single precipitin line against rat liver homogenate and 8 μ l of the absorbed antiserum precipitated dihydropyrimidine dehydrogenase activity of 0.02 g rat liver (about 0.48 nmol/min). Dihydropyrimidine dehydrogenase blotted onto membranes was developed by the use of the enzyme-labeled antibody technique. The result is shown in Fig. 2. The band of dihydropyrimidine dehydrogenase from vitamin B₂-deficient livers was weaker than that of controls. The enzyme levels from vitamin B₂-deficient livers decreased. Despite the lowering of dihydropyrimidine dehydrogenase as a result of vitamin B₂-deficiency, uridine, uracil and the Σ UMP pools in the liver were not

Fig. 1. Effect of vitamin B₂ deficiency on dihydropyrimidine dehydrogenase in rat liver. Four-week-old rats were given a vitamin B₂-deficient diet or a control diet for 30 days. Total liver flavin and enzyme activities were measured. * $p < 0.05$, ** $p < 0.01$: Significantly different from control. Each value is the mean $\pm$ SE from 5 rats. C, control diet; -B₂, vitamin B₂-deficient diet.

Table 3. Effect of flavin addition on liver dihydropyrimidine dehydrogenase activity.

Treatment	Final concentration of flavin (mM)	Dihydropyrimidine dehydrogenase activity (nmol·min ⁻¹ ·mg protein ⁻¹)	
		Control	Vitamin B ₂ -deficient
Exp. 1			
No addition	—	0.124±0.0055	0.042±0.0040**
FAD	0.5	0.034±0.0040	0.017±0.0044**
FMN	0.5	0.073±0.0073	0.033±0.0018**
Exp. 2			
No addition	—	0.083±0.0045	0.015±0.0064**
FAD	0.05	0.057±0.0073	0.012±0.0065**
FMN	0.05	0.053±0.0076	0.008±0.0037**

Exp. 1: FAD or FMN was added to the assay mixture and the enzyme activity in control or vitamin B₂-deficient rat liver at 37°C for 20 min was measured. Exp. 2: FAD and FMN was added to the homogenate, and incubated at 37°C for 30 min. Then the enzyme activity was measured. Each value is the mean±SE of five separate experiments. **p*<0.05, ***p*<0.01 compared to control.

Fig. 2. Western blotting of dihydropyrimidine dehydrogenase in vitamin B₂-deficient or control rat liver. Two samples represent random variables for each group. C, control diet; -B₂, vitamin B₂-deficient diet; E, purified enzyme (2.5 ng).

changed (Table 4).

DISCUSSION

The molecular weight of rat liver purified enzyme was 207,000 and was composed of two similar subunits. The enzyme contained 2 mol of flavin nucleotide

Table 4. Effect of vitamin B₂-deficient diet on uracil, uridine, and Σ UMP pool in rat.

Diets	Uracil	Uridine (nmol·g liver ⁻¹)	Σ UMP	Dihydropyrimidine dehydrogenase activity (nmol·min ⁻¹ ·mg protein ⁻¹)
Control	12.6±2.92	24.0±2.92	955±74.8	0.194±0.011
Vitamin B ₂ deficiency	13.2±0.75	22.5±2.21	926±31.1	0.047±0.009**

Each value is the mean±SE of five separate experiments. ** $p < 0.01$ compared to control.

and 14 mol of iron per mol of enzyme. The ratio of FAD to FMN was 1.0. The enzyme was originally purified from rat liver by Shiotani and Weber (6), and our data concerning the molecular weight of the enzyme were similar to their values. However, our results differ in that the flavin and iron content were not similar, as they reported that the enzyme contained 4 mol of FAD and 3 mol of iron per mol of enzyme. The enzyme described in this paper contained about 1 mol of FAD and 1 mol of FMN per mol of enzyme by an HPLC. FAD and FMN may combine respectively to subunits which have similar molecular weight but which are different. The difference between our data and that of Shiotani and Weber (6) may result from the difference in the method of flavin measurement. They determined the flavin content from the absorbance at 450 nm, but the absorbance at 450 nm may coincide with additional absorbance in the visible region which may be due to an iron-sulfur center.

Dihydropyrimidine dehydrogenase from pig liver was recently purified by Podschun *et al.* (22). The pig liver enzyme had a molecular weight of 206,000 and was apparently composed of two similar subunits. The pig liver enzyme contained 4 mol of flavin nucleotide and 32 mol of iron per mol of enzyme. Flavin released from the pig liver enzyme was identified as FAD and FMN in equal amounts. The absorption ratio (A_{269}/A_{432}) is 3 for pig liver purified enzyme. We found that the absorption ratio was 6.6 for rat liver purified enzyme. Thus the rat liver enzyme may be in a half-reduced form due to elution with NADPH from ADP-Sepharose, while the pig liver enzyme was eluted with KCl. Dihydropyrimidine dehydrogenase may be species specific, since antiserum to rat liver enzyme did not precipitate dog or guinea pig liver enzyme activity. Iron tightly bound to the enzyme and could not be removed by EDTA or other chelating reagent treatments. However, the accurate content of flavin as well as iron in native dihydropyrimidine dehydrogenase should be determined by further detailed studies.

On the other hand, the roles of flavin bound to the enzyme are thought to be follows: 1) an electron acceptor or donor; 2) to maintain the active structure of dihydropyrimidine dehydrogenase; 3) to regulate the degradation rate or the synthesis of dihydropyrimidine dehydrogenase *in vivo*. In the case of glutathione

reductase in erythrocytes, holoenzyme decreases and apoenzyme increases by vitamin B₂ deficiency (23). Addition of FAD recovers the activity *in vitro* and total enzyme activity with flavin does not change. So in erythrocytes, flavin is related to glutathione reductase enzyme activity and it does not change half-life of the enzyme. However, it was suggested that flavin regulates the degradation rate or the synthesis of dihydropyrimidine dehydrogenase *in vivo*, in liver, because of the decrease in dihydropyrimidine dehydrogenase activity (Fig. 1) and the decrease in enzyme levels (Fig. 2) in vitamin B₂ deficiency.

Smith and Yamada (24) reported that NADPH-dependent uracil hydrogenase activity was distributed mainly between mitochondria or plasma and endoplasmic membranes. But we found that the enzyme was located in the cytosolic fraction similar to other pyrimidine degrading enzymes. It is thought that uracil degrades to β -alanine in cytosol and β -alanine is transported into mitochondria, and further degraded by aminobutyrate aminotransferase (10,25) and D- β -aminoisobutyrate-pyruvate aminotransferase (25). However, in spite of the lowering of dihydropyrimidine dehydrogenase activity in vitamin B₂ deficiency, Σ UMP, uridine and uracil content in liver did not change. It is well known that pyrimidine pool is regulated by its *de novo* synthesis and salvage synthesis. Thus *de novo* synthesis and salvage of pyrimidine may have a more important role than pyrimidine degradation in the regulation of the pyrimidine pool in vitamin B₂-deficient rats.

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